Visualizing Cells and Humans in 3D

Biomedical Image Analysis at Nanometer and Meter Scales

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Researchers have analyzed and presented volume data from the Visible Human Project and data from 3D ion-abrasion scanning electron microscopy. Despite the two datasets' difference in subject and scale, the methods for analyzing and modeling them are remarkably similar. They are derived from image-processing, computer vision, and computer graphics techniques.

More than 15 years ago, the Visible Human Project (VHP) announced the most detailed study of human anatomy ever attempted.[1] The study employed emerging technology in digital photography and cryosectioning, a destructive imaging technique that achieves pixel resolutions on the order of 300 microns across subjects nearly 2 meters long. The resulting datasets exceeded 17 Gbytes and represented a landmark in the medical sciences.

Today, the High Performance Computing and Communications Office and the Audiovisual Program Development Branch of the US National Library of Medicine and the Laboratory of Cell Biology at the US National Cancer Institute are teaming up to investigate biomedical sciences at nanometer scales using ion-abrasion scanning electron microscopy (IA-SEM). IA-SEM is an emerging technology for 3D imaging of whole cells and tissues at resolutions in the 10-nm range.[2,3]

Despite the difference in subject and scale across the subject matter, the methods for analyzing and modeling them are remarkably similar. They are derived from image-processing, computer vision, and computer graphics techniques. Our process reduces large data to a manageable computational model suitable for quantitative analysis while promoting abstract concepts of shape, structure, and function. Moreover, we are employing medical illustration, visualization, and rapid prototyping to inform and inspire the biomedical sciences. By combining graphics and biology, we are imaging biologically relevant objects at the nano and macro scales to improve public health through research.

Scope and Scale

There are several ways to describe the resolution of a volumetric digital image. *Resolution* often relates to the number of pixels of an image. *Field of view* usually refers to the size or dimensions an image spans in physical space. Regarding data acquisition, other concerns arise. For this article, we'll call the subject matter's field of view the image's *outer scale* and the minimum resolvable feature size the *inner scale*.

Inner scale is distinct from pixel resolution (or voxel resolution in 3D) because it's derived from the process or technology used in data acquisition. Factors influencing the inner scale of 3D data include the point-spread function of the imaging system, the sensitivity of the detector and its susceptibility to noise, and artifacts and variations of the subject material. The size of the spot that the electron beam creates, the precision of the microscope's objective lens, and even asymmetries in the data collection affect inner scale. Even if a microscope can generate an

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Case Study 1: The Scale of Mice and Men

Under the Visible Human Project,[1] the University of Colorado Health Sciences Center created a custom cryomacrotome using a specially constructed 14-inch diameter disk with 20 hardened teeth around its perimeter. The milling blade remained in a fixed position operating at 300 rotations per minute, and the subject block was moved under the cutting head and elevated between slices. Figure A gives an overview of this process.

The researchers carefully selected two cadavers from human subjects who donated their bodies to science. Before milling or sectioning, they perfused the cadavers with approximately 19 liters of formalin (a chemical fixative to arrest all latent biological activity). Later, they immobilized and eventually froze the subject. Because

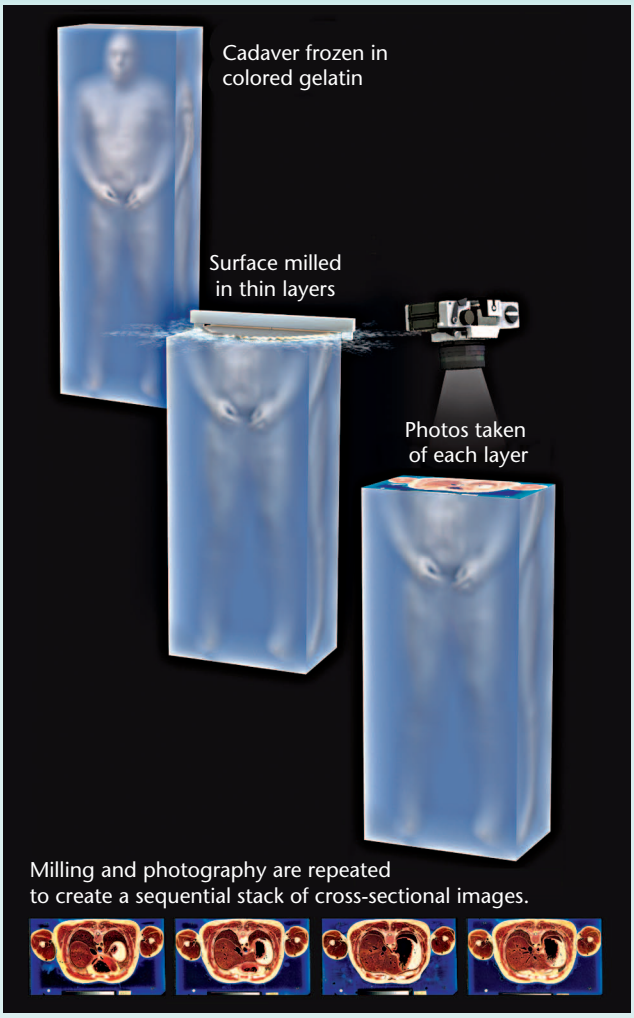

Figure A. The Visual Human Project (VHP) data collection process. Researchers first froze and embedded cadavers in ice. They incrementally carved the resulting sample using a custom milling machine, exposing layer after layer of human anatomy. They photographed the freshly planed block face with submillimeter precision. They did not apply staining or perform digital tissue classification. The RGB values of the images reflect the fixed tissue's natural colors. Researchers used these techniques to perform a comprehensive study of human anatomy on a male and female subject. They generated thousands of sequential digital images per subject that constitute a whole-body volume.

image with pixel dimensions measured in nanometers, the inner scale of the image will be measured in microns if the focus of the objective lens or the indistinct glow of fluorescent proteins only allows the capture of micron-sized features. The drive for more magnification is limited by physics, optics, scanning speed, and the ability to focus photons or electrons and ions.

The outer scale of an image is usually limited by the extent and cost of the detector and is often in direct opposition to improvements in the corresponding inner scale. Decreasing a microscope's objective power or zooming out with a camera's optics will increase the outer scale at the expense of the inner scale. Regarding these trade-offs, biomedical sciences have a driving need to work at the scope of whole organs, the relationships between entire cells, or whole-body images. Simultaneously, they need to see these entire subjects at the highest-possible magnification.

Modern microscopy is rapidly adopting digital methods to adapt and combat these trade-offs. Digital registration enables large composite mosaic micrographs from multiple images, effectively increasing the outer scale without sacrificing magnification.[4]

Block-faced imaging microscopy methods record entire cell populations while capturing extremely fine details.[5] These methods generate large datasets, requiring software developers to analyze data that often will not fit in main memory. Our investigations with the VHP, its large datasets, and its associated software development programs helped us tackle microscopy problems with minor adaptation of our techniques. We compare the application of our methods to whole human subjects with an outer scale of 2 meters to those same methods applied to voxel data of single cells with an inner scale of 15 nm.

Common Ground in the Digital Domain

The two case studies in this article (see the sidebars) represent some of the most advanced 3D-imaging

of the physical limitations of the sectioning apparatus, they partitioned the embedded subject into blocks no larger than 56 × 53 × 36 cm. They embedded each cadaver section in a matrix of 3 percent gelatin colored blue with food dye and transferred it to a freezer (–85°C) for at least 12 hours.

Using the custom cryomacrotome, the researchers incrementally milled each block section as slices at millimeter scales. They then imaged the resulting exposed surfaces with some of the most sophisticated digital photography equipment available. A Hasselblad 553 ELX camera body with a 2,048 × 2,048 × 14-bit Leaf camera back mounted with a Zeiss Distagon f4 50-mm lens set to f6.8 with a polarizing filter generated a field of view of 63.5 × 63.6 cm. An RGB color wheel created three color channels.

Ultimately, the male dataset comprises 1,878 slices of 2,048 × 1,216 voxels. Voxel resolutions are 1 mm and 0.33 mm in the *z*-direction for the male and female datasets, respectively, and 0.32 × 0.32 mm in the *xy*-plane. Figure B shows example renderings.

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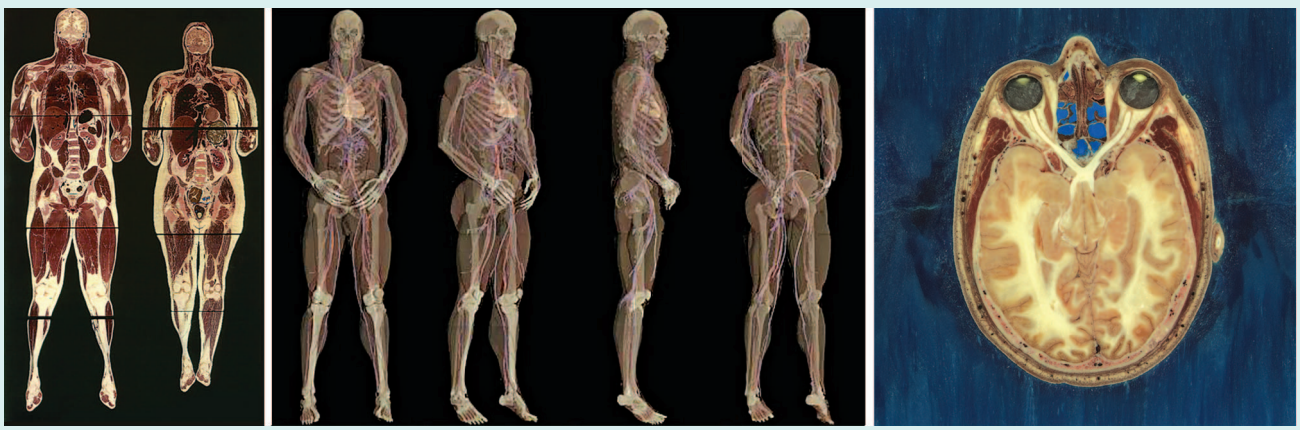
1. V. Spitzer et al., "The Visible Human Male: A Technical Report," *J. Am. Medical Informatics Assoc.*, vol. 3, no. 2, 1996, pp. 118–130.



(1) (2) (3)

Figure B. VHP renderings. (1) Coronal views. (2) Volume renderings. (3) An axial-image close-up of the head. The VHP collected whole-body data of two human subjects, male and female, at millimeter resolutions using a custom cryomacrotome. The resulting datasets remain some of the most comprehensive studies of human anatomy today.

approaches of biomedical subjects. Although these studies deal with vastly different scales, their inherent basis in milling and block-face imaging lead to similar computational approaches. The deformable biological subject matter creates similar needs for the samples to be fixed, immobilized, and embedded before researchers can capture the data.

Preprocessing

The common theme of incremental slices for data capture causes cryosectioning and IA-SEM to have similar data-preprocessing requirements. The pixels of each slice are acquired simultaneously, but conditions between slices vary in small ways.

There are many factors that create variations in image position and color in cryosectioning. The cryomacrotome system (see the "Case Study 1" sidebar) has mechanical systems for carefully positioning the frozen blocks under the digital camera. However, it's not feasible to precisely, accurately, and repeatedly stop a mass of ice exceeding 106 kg within 100 microns during thousands of trials. Despite consistent dressing of the exposed surfaces with dry ice between milling, over the course of a day, the rising temperature of the cryomacrotome will affect the images' color. Daily power and ambient-temperature fluctuations can also affect the digital detector's sensitivity.

In IA-SEM, several factors can affect the alignment and exposure consistency of the captured digital images. When we are trying to achieve pixel resolutions of 3 to 6 nm, even slight changes in temperature affect the detector's sensitivity or induce thermal drift, affecting spatial alignment.

Whether the source is a cryomacrotome or IA-SEM, digital preprocessing has similar mathematics. Alignment in the *yz*- or *xz*-planes is seldom, if ever, required, but *xy*-registration of stacked slices is mandatory.

In the VHP data the research team placed fiducial markers in the frozen gelatin to aid in alignment. With IA-SEM data, we use commercial and

Case Study 2: 3D Imaging at a Cellular Scale

Our multidisciplinary team has been developing new methods in high-resolution electron microscopy to capture volume datasets containing entire cells. In a process that corresponds broadly to the cryomacrotome, we use a system equipped with a gallium ion source for focused ion beam milling. We also use a field-emission-gun scanning electron microscope (SEM) with an in-lens secondary electron detector for imaging to incrementally acquire image stacks comprising 3D volumes of cellular samples. The focused ion beam is capable of milling and polishing with nanometer precision.[1] Figure C gives an overview of this process.

Typically, we prepare cell samples of interest for imaging by adding a fixative buffer of glutaraldehyde in a sodium cacodylate solution, arresting all biological activity. After postfixing with 1 percent osmium tetroxide (OsO_4) in a 0.1 M (molar volume) sodium cacodylate buffer and stained with 0.5 percent uranyl acetate in a 0.1 M acetate buffer, we dehydrate the samples through graded ethyl alcohol followed by propylene oxide. Samples are then infiltrated overnight at room temperature with a 1:1 mixture of epoxy resin and propylene oxide and cured for 48 hours in an oven at 55°C. We trim the resulting samples into blocks with 2-mm surface areas.

Using the focused ion beam, we incrementally mill the sample blocks containing whole cells (with a typical interval between layers of 10 to 20 nanometers). We image the resulting exposed surfaces with the SEM directly from the face of the remaining block. Secondary electron SEM im-

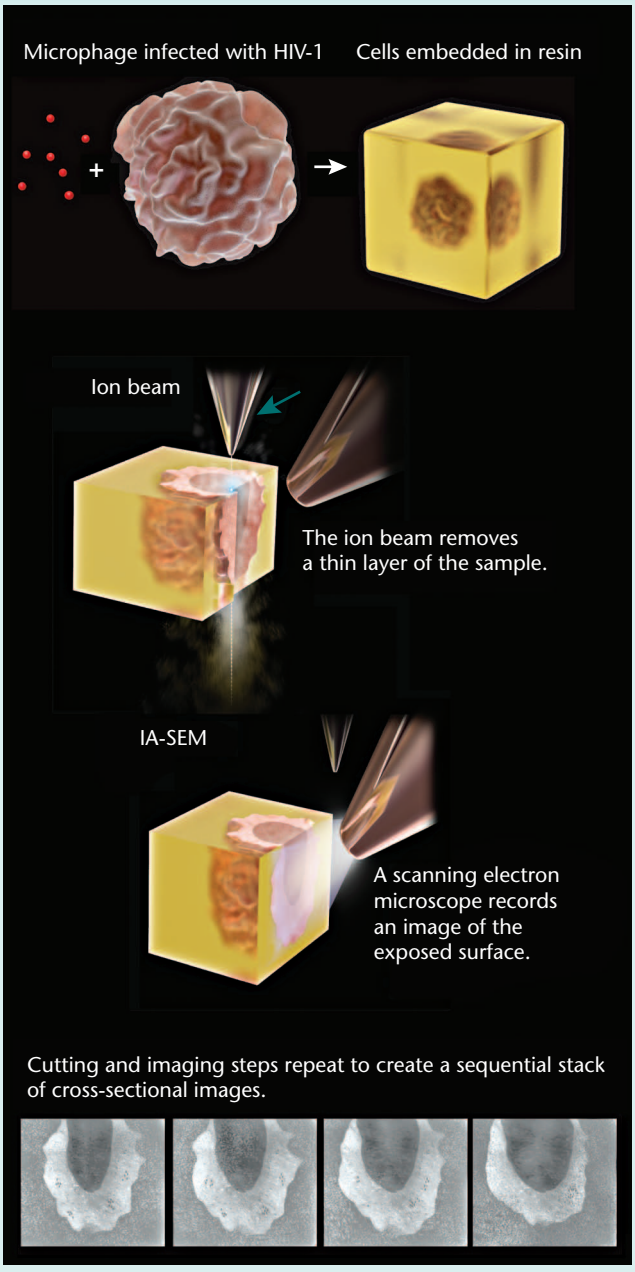


Figure C. Data capture with ion-abrasion scanning electron microscopy (IA-SEM). We prepare samples using techniques similar to those for transmission electron microscopy, embedding the fixed and dehydrated biological sample in an epoxy resin. An ion beam repeatedly mills or abrades and polishes a flat surface in the sample, exposing layer after layer of structure at a cellular level. A scanning electron microscope acquires images of the exposed faces of the sample block. Each pass of the ion beam can remove layers as thin as 10 nanometers, with pixel dimensions as small as 3 to 6 nm.

open source software packages to align the image stacks. We manage brightness and intensity correction by analyzing the histogram of the image intensities and creating an algebraic correction based predominantly on the *z*-value of the slice. Another characteristic of both imaging modalities is that the imaging system dominates the point-spread function in the *xy*-plane. In the *z*-direction, other factors such as electron or photon penetration create an asymmetric response along the *z*-axis. Proper filtering, averaging, and denoising of such data should take this into account and will result in asymmetric, anisotropic methods.

Segmentation

Both modalities generate relatively large data volumes. The size of these datasets overwhelms many image segmentation software packages. Often, research teams must therefore write custom software to accommodate large 3D data. One exception to this is the Insight Segmentation and Registration Toolkit (ITK). Originally funded by a consortium of US National Institutes of Health and other federal agencies, the software architecture of this open source, publicly available library was designed for streaming processing, permitting out-of-core data analysis. Researchers recently demonstrated

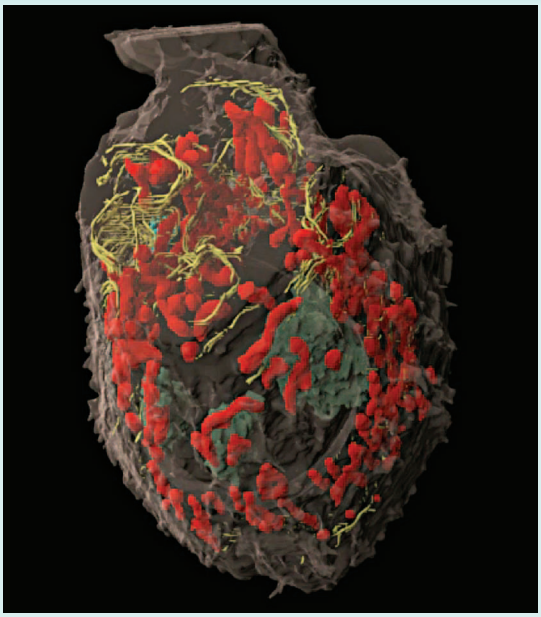


Figure D. A rendering of a human melanoma cancer cell from data acquired using IA-SEM. This view clearly shows the relative placement of organelles such as mitochondria (red) and the endoplasmic reticulum (yellow). Individual voxel dimensions are 6 nm in the *xy* direction and 20 nm in the *z* direction.

ages are typically recorded at accelerating voltages of 1 to 3 kV, 10,000× magnification, and a beam current of 68 to 270 pA (picoamperes) in the immersion lens mode. Depending on the sample, each dataset can contain a stack of as many as 1,000 images. Figure D shows an example rendering.

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1. J.A. Heymann et al., "Site-Specific 3D Imaging of Cells and Tissues with a Dual Beam Microscope," *J. Structural Biology*, vol. 155, no. 1, 2006, pp. 63–73.

and made publicly available the source code for filtering large datasets, including I/O support of the VTK (Visualization Toolkit) and MRC (Medical Research Council) microscopy file formats.[6]

Figure 1 illustrates how we applied ITK methods to IA-SEM data. The sample is an HIV-infected monocyte-derived macrophage, part of the immune system. Macrophages can harbor HIV, inadvertently acting as reservoirs of the infection. We analyzed the captured data to evaluate and observe the disposition of HIV in the infected cells.[7]

Analysis of a large dataset involves two essential requirements. First, you must understand the resolution and scale needed to accomplish the task. Second, you must use the simplest algorithms available to accomplish the task, because large data presents a computational burden that can make an approach unusable.

To preprocess the macrophages, we used a modified curvature diffusion equation to align and denoise the data.[8] We observed that the cells' interiors were heterogeneous but that the exterior resin had a constant intensity (except for imaging artifacts). Additionally, the cells contained enhanced boundaries in many places but weaker ones in others.

For segmentation, we first applied, with manually selected seeds, threshold-based region growing. As we expected for this simplistic approach, the chosen threshold either leaked into the cell or undersegmented the region. We used the undersegmented region as an initial condition for a level-set method containing an expansion term and a mean curvature penalty.[9] Owing to the observed strong edges, a sigmoid function of the gradient magnitude drove the expansion term. The complement of the resulting level set contained the cell and the virus.

Although viruses have varying sizes and appearances based on their maturity, they are still essentially spherical. The Hough transform detects sphere-like objects by having feature points "vote" in a 4D parametric space. The naïve implementation of the transform is expensive in both computation and memory. To reduce the memory required, we reduced the radius dimension in the parametric space by maintaining the average radius for a given center. To reduce the computational expense, we selected quality points, which lie on the optimal edge, on the basis of John Canny's maximum gradient magnitude criterion.

Finally, we manually segmented the interior viral channels and pockets. We used binary operations and morphology to exclude the virus channels and the viruses from the level-set mask, as well as to exclude false-positive virus detections in the cell.

We found HIV congregating in pockets throughout the cell. Figures 1c and 1d compare the compartments where HIV is sequestered in the macrophage. In the 2D view, the compartments appear to be bubble-like vesicles, whereas the 3D view shows that the compartments are physically contiguous with the extracellular environment.

Visualization and 3D Printing

Although algorithms and techniques for rendering volume datasets are straightforward today, presenting large data remains a computational challenge

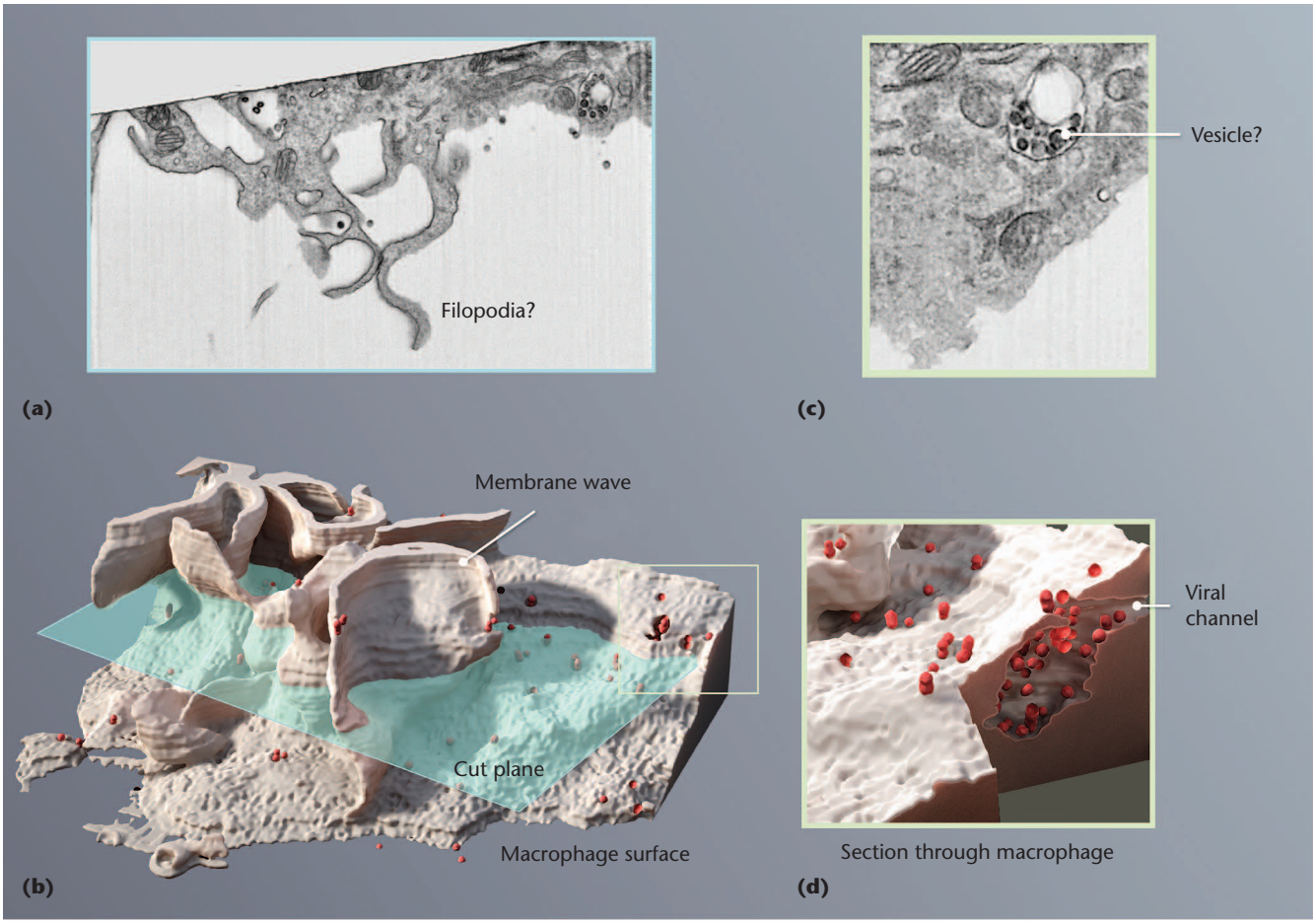


Figure 1. Results from ion-abrasion scanning electron microscopy (IA-SEM) of HIV-infected monocyte-derived macrophages. (a) 2D imaging can lead to the erroneous conclusion that the structures of interest are filopodia (thread-like filaments of membrane). (b) 3D imaging reveals that these structures are membrane waves or walls. (c) 2D imaging suggests that HIV virions are sequestered in vesicles or bubbles in the cell. (d) 3D imaging reveals that the structures are not vesicles but are pockets that have complex tunnels and channels that communicate with the environment outside the cell.

requiring complex data management and software design. We use a variety of techniques to render our data. Figure 2 shows feedback loops in our data flow among segmentation, visualization, and quantitative measurement.

A relatively new addition among our research tools is a 3D-printing, rapid-prototyping, layered manufacturing system that renders full-color inkjet representations as plaster 3D models.[10] (Figure 3 shows our first attempt to apply this tool to biomedicine using VHP data.) We directed this technology toward high-resolution electron microscopy. In one study, the team examined the mechanisms by which HIV virions are transferred from dendritic cells to T cells during cell-to-cell contact. Two-dimensional transmission electron microscopy studies show that T cells are encircled by cytoplasmic processes emanating from the dendritic cell. However, 3D microscopy shows that, rather than form a lasso-like structure, the dendritic cells create cradle-like structures out of sheets or ruffles of membrane that embrace the T cell. These structures contain and perhaps control the local chemical and biological environment around the T cell, accelerating pathogen transfer between them.

When we first considered how to portray this observation, we reconstructed the data from IA-SEM as a solid model in a series of four slabs. We segmented the T cells and dendritic cells from the surrounding serum or background using level sets and mathematical morphology. We colored the T cells red or pink to help discriminate the two cell populations. We converted the resulting data into textured isosurfaces and printed them with the 3D printer. This provided valuable insights into the spatial arrangement of membranes in the cell-to-cell contact region (see Figure 4).[11]

Quantitative Measurement

As we traverse the image analysis pipeline, we progressively reduce the size of the data stream while building meaningful abstractions about the information it contains. A continuing goal is to

enable quantitative studies about the shape, size, geometry, and structure of the specimen. This step beyond qualitative observation of biological phenomena promotes a range of science that can employ and build on established tools of statistics and mathematics. We must derive metrics and measurements from our data that can leverage these mature disciplines.

Figure 5 illustrates quantitative measurement in our research. Methylmalonic acidemia (MMA) is a lethal congenital dysfunctional metabolic condition. Researchers have noted that mitochondria, organelles responsible for cell respiration, are unusually large and distorted in subjects afflicted with MMA. In a comparative study, we collected 3D data volumes from normal and diseased tissue samples using IA-SEM. Using our data analysis approach, we segmented and analyzed the mitochondria in both populations.[12]

We took liver cells from normal and MMA-afflicted mice. We prepared the tissue and collected volume images with IA-SEM. We manually segmented the mitochondria from both sample populations and discarded partial organelles. Then we analyzed the remaining mitochondria and computed the Gaussian curvature across the membrane.

The distorted shapes of the MMA-afflicted mitochondria are visually apparent, whereas the normal mitochondria appear as smaller, simpler spheroids. A spheroid has only positive Gaussian curvature, while bifurcating shapes have relatively large hyperbolic patches with negative Gaussian curvature. To quantify the extent of the distortions using a metric that is invariant to changes in size, we measured the percentage of positive Gaussian curvature for each mitochondrion. We plotted this value for both populations; the results suggest that mitochondrial shape is possibly an early indicator of this disease (see Figure 6).

Discovery in Three Dimensions

The technologies for generating 3D data of intracellular subject material have emerged only in recent years. The case for both capturing data and modeling information in three dimensions (and higher-dimensional spaces) is compelling. Life exists in three dimensions, and understanding the spatial relationships among the features and phenomena requires modeling and analysis beyond 2D space.

Figure 1 demonstrates this idea. *Filopodia* are filamentous or thread-like extrusions of the cell membrane that macrophages can use to snare and envelope viruses and other pathogens as a defensive part of the immune system. Our IA-SEM studies have revealed new insights into the membrane organization on the surfaces of macrophages. Features that might be considered filament-like in two *xy* dimensions actually have broad extent in the *z* dimension, creating wall-like structures rather than finger-like projections. These massive

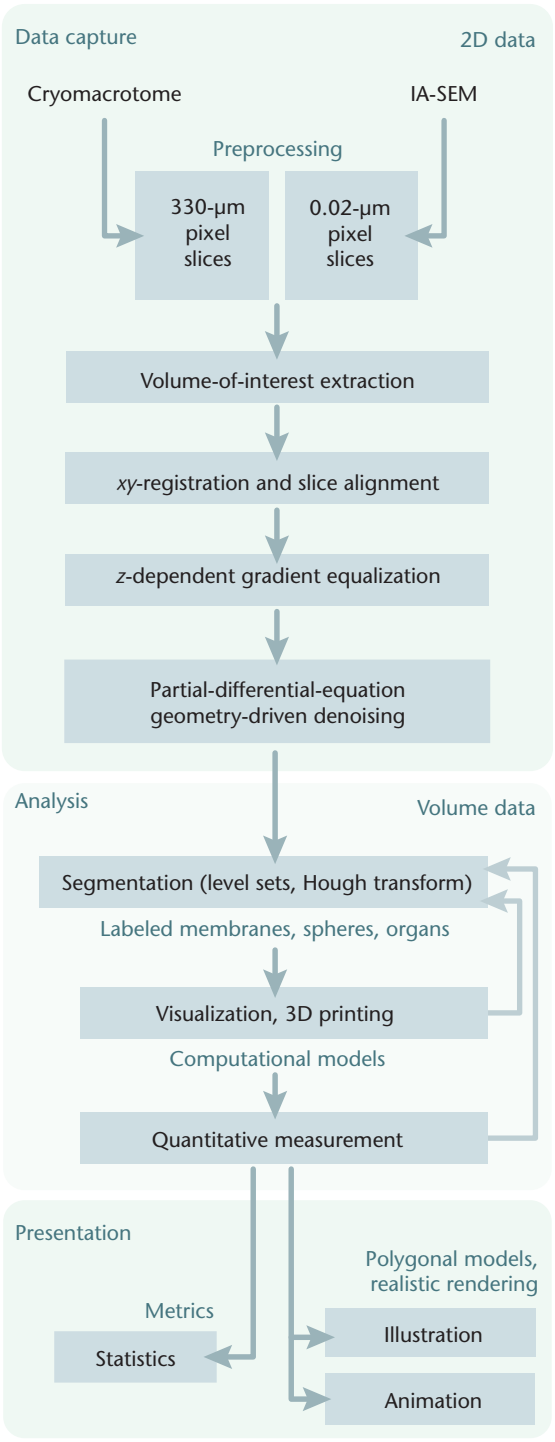
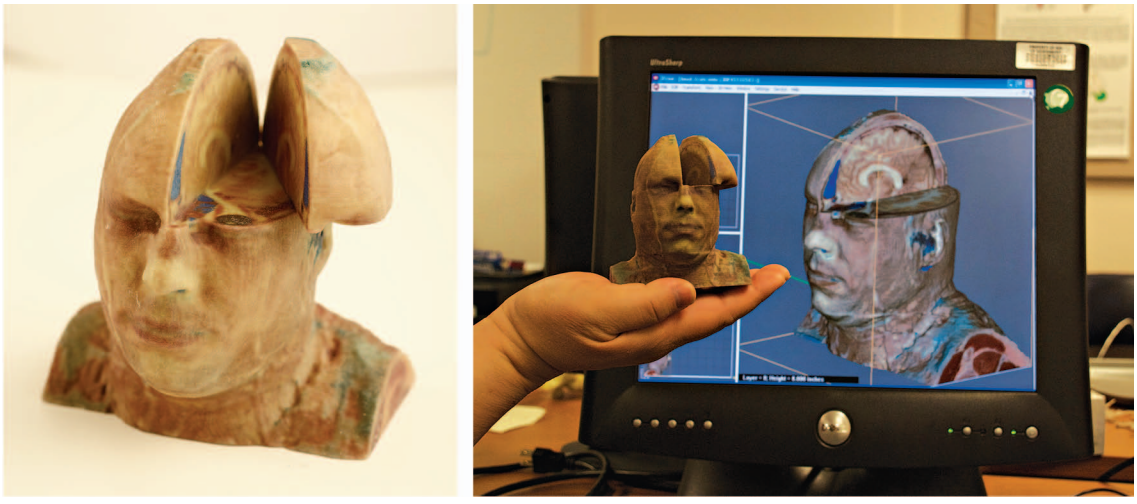


Figure 2. The shared data-processing pathway for cryosectioning and IA-SEM. The overall trend from data collection to generated models is to increase levels of abstraction while reducing data from Gbyte volumes to Kbyte mathematical, statistical, or computational models.



(a) (b)

Figure 3. Applying 3D printing to our research. (a) We created this printout of the Visual Human Project male head. (b) Commercial printing software creates raster scans from polygonal models, generating incremental layers printed with inkjets to build solid models from plaster, ink, and liquid binder.

(a) (b) (c)

Figure 4. Cell-to-cell contact between dendritic cells and T cells. (a) A 2D transmission electron microscopy image of a T cell surrounded by dendritic cells. (b) A 3D print of IA-SEM data. T cells are red; we re-created the volume as slabs. We used level sets and mathematical morphology to segment the cell surfaces. (c) A rendering of the cradle-like structures. We digitally removed two T cells in the foreground to reveal the complex underlying structure.

microscopic wave-like projections could be far more efficient than filopodia at particle capture. These membrane extensions likely correspond to the "ruffles" or "veils" we observed through SEM of the macrophage surface.[3]

Focusing on Presentation

Volume data created with IA-SEM is so dense with detail that figure creation begins with deciding and planning the focus. Instead of including layers of beautiful, rich data, each figure's core message dictates what will be omitted for clarity's sake. We can omit structures or render them transparently while resegmenting other crucial details with greater care.

We translate membranes, organelles, or other structures into polygonal models. We then manipulate them with 3D software such as Autodesk 3D Studio Max or Maya. We often use additional rendering plug-ins (such as SplutterFish Brazil or Autodesk Mental Ray) for specific effects to create a visual hierarchy of importance.

To further focus the view, we employ various effects. Color creates emphasis. Realistic shadows add a layer of realism that helps viewers understand compound overlapping forms. Materials such as translucent ray-traced glass show both a surface and what is obscured beneath. Radiosity helps mimic real-world lighting, enabling viewers to more easily perceive and comprehend complex organic shapes. Finally, subsurface scattering allows suggestions of volumes such as the thin structures of membranous folds. Each additional effect clarifies the message while reducing the clutter of nonrelated information.

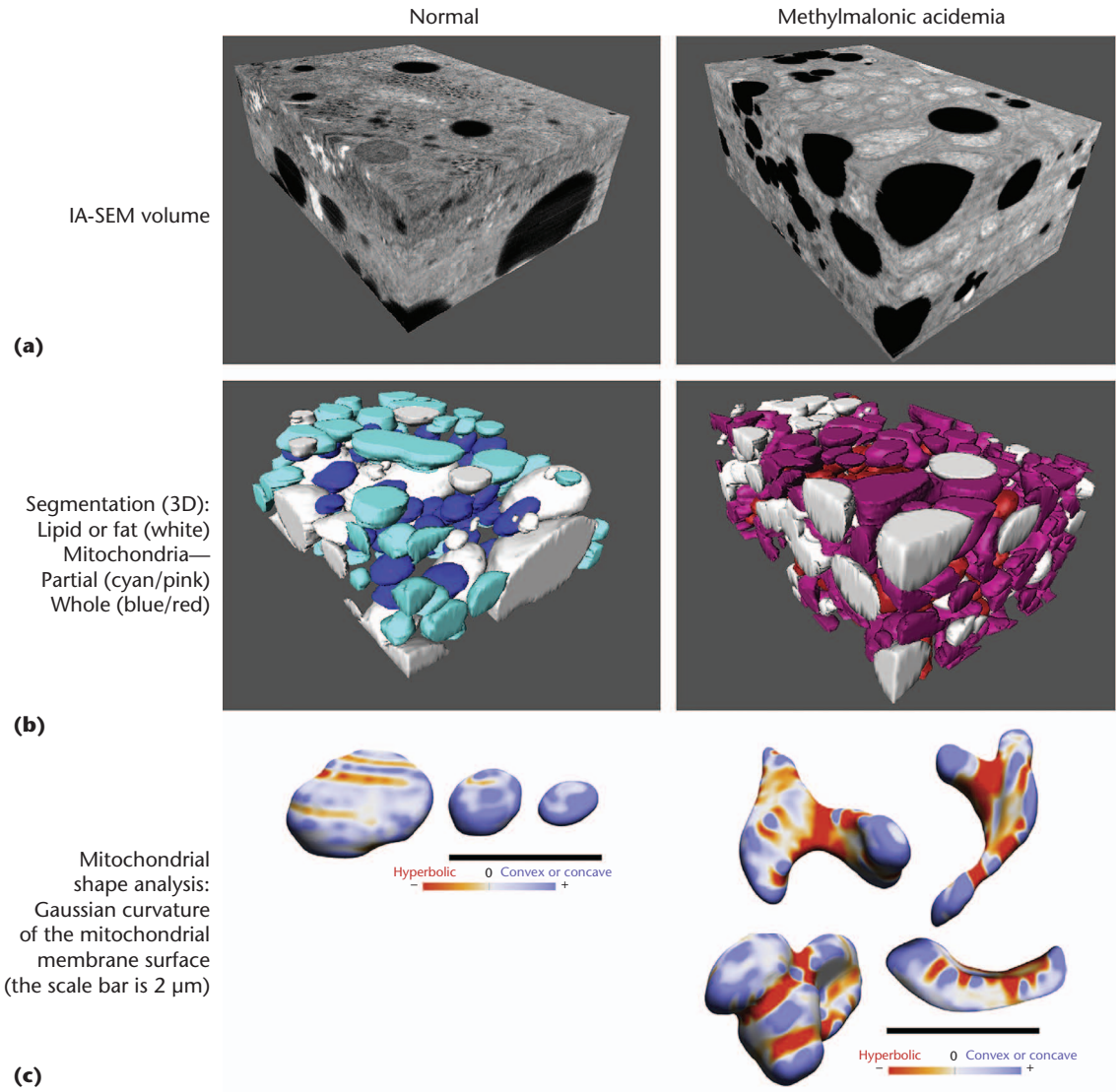


Figure 5. Comparing the mitochondrial geometry of normal tissue (left column) and tissue from a subject with methylmalonic acidemia (MMA; right column). (a) 3D IA-SEM of liver cells. (b) Manual segmentation and classification of mitochondria and lipid vacuoles. (c) Shape analysis of Gaussian curvature across the mitochondria's surface. (Normal mitochondria are predominantly elliptical; MMA-affected mitochondria are distorted.)

We're only beginning to leverage the capabilities of our current technology to explore the life sciences at nanometer scales. With this drive to see smaller structures, we face problems of increasing noise and artifacts in the signal as we accelerate the speed of data acquisition; quantum physics events are also a significant consideration. Moreover, the increasing sizes of collected datasets pose computational questions that must be answered.

Other challenges include merging different imaging modalities. Radiology researchers routinely fuse positron-emission-tomography data, which is noisy, relatively low-resolution data on physiology, with higher-resolution x-ray computed-tomography data, which has good anatomical content, to leverage each modality's best capabilities. Likewise,

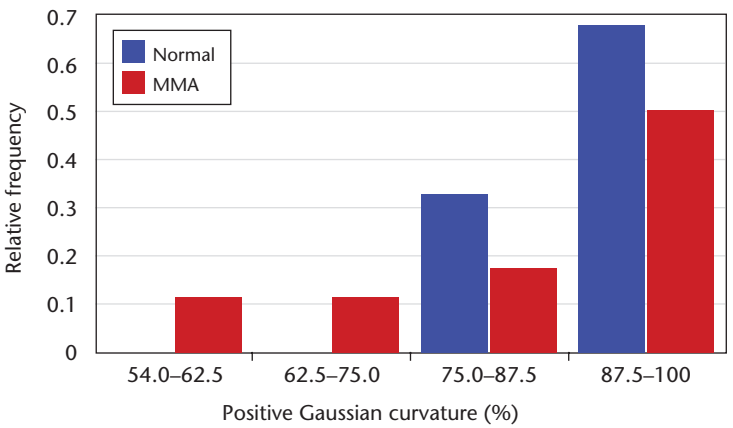


Figure 6. Plotting the percentage of positive Gaussian curvature yields a size-invariant metric that implies two distinct populations. This suggests that mitochondrial shape is possibly an early indicator of MMA.

correlative imaging combines fluorescent confocal and two-photon microscopy with IA-SEM. On the basis of our successes in repurposing software, we hope to adapt existing methods developed for 3D fusion of multimodal radiological data to microscopy. The goal in correlative imaging is multimodal registration of biochemical information from relatively noisy, low-resolution confocal data with structural information from IA-SEM at nanometer scales.

Discovery today in science and medicine relies on computing and other advances in technology. Disciplines such as radiology, microscopy, and even astronomy are possible only through a blend of the technologies for data capture and data analysis. Before now, experts in gross anatomy and microbiologists had little common ground. Today, however, as computer scientists are joining multidisciplinary research teams, they are linked by software and supporting data analysis practices.

The evolution of whole-body imaging and whole-cell high-resolution electron microscopy projects exposed many shared software concerns in each of them, despite a dramatic difference in the posed problems' physical scales. Ultimately, the shared process of volume image analysis is one of data reduction, decreasing the problem's dimensionality into quantifiable metrics that lead to rigorous scientific study. Early investment in segmentation and registration algorithms as well as a strong software process will hopefully enable us to continue to rapidly adapt our current methods in radiology to enable new discoveries inside individual cells.

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